APPLICATION NOTE

Sequence and Side-Chain Specific Photofragment (193 nm) Ions from Protonated Substance P by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

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Photodissociation at 193 nm (6.43 eV) of the protonated substance-P, $[M + H]^+$ ions, in a delayed extraction matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometer, is reported. The photofragment ion spectrum of substance P contains a complete series of a-type fragment ions and abundant side-chain cleavage ions. This article focuses on the utility of MALDI-TOF photodissociation for peptide sequencing. (J Am Soc Mass Spectrom 1999, 10, 1038–1040) © 1999 American Society for Mass Spectrometry

Matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry is an established method for characterization of polar macromolecules, especially peptides and proteins [1]. The increased mass resolution achieved by delayed extraction (DE) [2] greatly improved the analytical utility of MALDI-TOF MS, especially instruments equipped with a reflectron [3]. The combined use of DE and reflectron TOF instruments permits high mass resolution (>10,000) and high mass measurement accuracy (<10 ppm) for peptides having molecular weights of up to approximately 7–8 kDa. The increased mass resolution and mass measurement accuracy afforded by DE-RTOF has proved especially beneficial for proteomics and direct analysis of complex protein mixtures by using enzymatic digestion and peptide mapping [4]. In this article, we focus on the added dimension of structural characterization of peptides by using MALDI-TOF. Numerous laboratories now use MALDI-R-TOF and post-source decay to examine fragmentation reactions of gas-phase ions, particularly for peptide sequencing [5]. The primary limitation of PSD peptide sequencing is that the internal energies of the $[M + H]^+$ ions are not sufficient to yield a complete series of sequence ions. In most cases, fragment ions corresponding to loss of two to three N- and/or C-terminal amino acid residues are detected but the abundance of additional fragment ions is quite low. It is possible to increase the total fragment ion yields detected by PSD by introducing collision-induced dissociation (CID); however, as the mass of the analyte ion increases, the yield for CID fragment ions decreases and mass shifts due to energy loss accompanying collisional activation reduce mass measurement accuracy.

An alternative method for effecting dissociation of the $[M + H]^+$ ion is photodissociation [6]. Photodissociation involves excitation of the $[M + H]^+$ ion by absorption of a UV-VIS photon, and the most effective photon source for this experiment is the pulsed lasers that are routinely used for TOF mass analysis. The amide linkage of peptides strongly absorbs at 193 nm; thus photodissociation by radiation from an excimer laser is highly compatible with PSD-TOF fragment ion detection modes. The absorption of a 193-nm photon deposits approximately 6.43 eV (148 kcal mol^{-1}) of excitation energy into the ion, and this amount of energy should be sufficient to dissociate the peptide at the amide linkage. Detection of photofragment ions by TOF methods then becomes dependent upon the kinetics of the photodissociation process. For example, the transient time of the ions from the point of photoexcitation to the entrance of the reflectron ranges from a few microseconds to several hundred microseconds; thus it is important that sufficient internal energy be imparted to the ion for the dissociation rate to be greater than 10^6 s^{-1}.

Experimental

Sample Preparation

Substance P and alpha-cyano four-hydroxy-cinnamic-acid were obtained from Sigma (St. Louis, MO) and used without further purification. Samples were prepared in 50 uL 2:1 water to methanol solution containing 1 uL of 1 mg/mL analyte in water, 16 uL of 6

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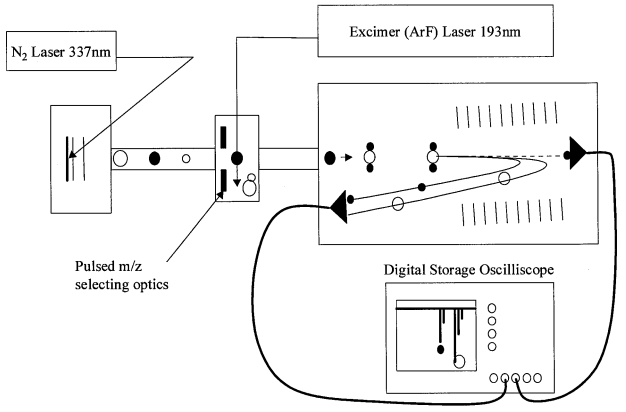

Figure 1. Diagram of home-built tandem TOF mass spectrometer used for photodissociation studies.

mg/mL matrix in methanol, and 33 uL of water. A matrix layer is made on the sample plate by spotting 1 uL of 32 mg/mL matrix in methanol and allowing it to dry. A 1-uL aliquot of the analyte/matrix mixture is deposited onto the dry matrix layer, and is allowed to dry. The analyte/matrix spot is washed with 5 uL of water to remove salts and is allowed to dry.

Mass Spectrometry

The PID spectrum of substance P was obtained using a home-built MALDI DE reflectron TOF (see Figure 1) that is described in detail elsewhere (manuscript in preparation). Briefly, the ion source contains a sample plate and two grids. The grids are used to define the electric fields and are positioned at distances of 3 and 20 mm, respectively, from and parallel to the sample surface. The excimer laser intersects the ion packet orthogonally at a distance of 0.5 m from the second grid of the ion source. The laser beam energy at the intersection region is approximately 500 uJ and is focused with a plano-convex lens resulting in beam dimensions at the intersection region of 5 mm $\times$ 0.25 mm and an irradiance of 2×10^6 W/cm^2. The timing between the nitrogen laser output and the excimer laser output is stable to +/− two nanoseconds [7]. The flight path distance from the ion/excimer intersection to the ground grid of the reflectron is 1 m. The total effective flight path distance is 3.5 m. The metastable/photofragment spectra are calibrated using eq 1 taken from page 7-6 of the Voyager Biospectrometry Workstation User's Guide. Equation 1 is a modified form of the standard PSD calibration described by Cotter [8].

$$m_f = m_p \frac{[(T_f/T_p) - C_1]}{(1 - C_1)} (\text{MirrorRatio} - C_3(1 - \text{MirrorRatio})^{C_4}) \quad (1)$$

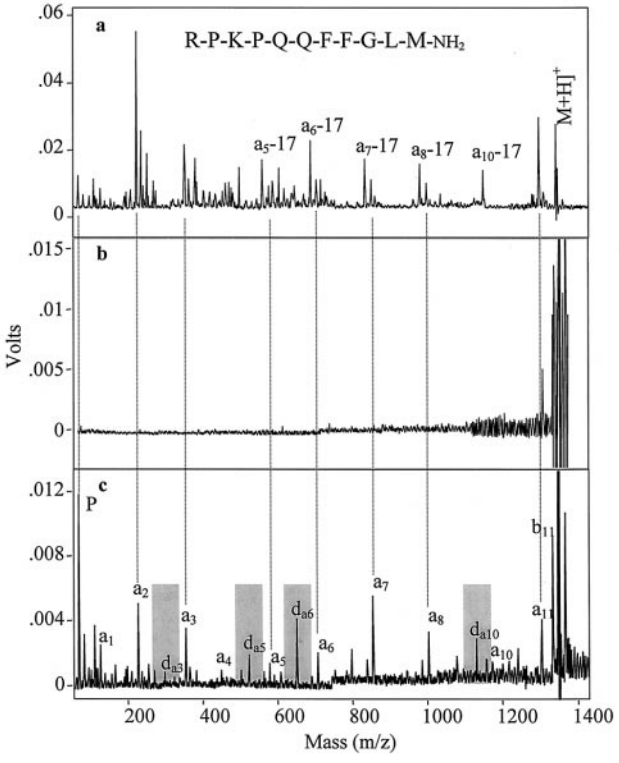

Figure 2. (**a**) Metastable (high MALDI laser power), (**b**) photofragment background (low MALDI laser power), and (**c**) photofragment spectra of substance P-NH_2 $[M + H]^+$ (low MALDI laser power). Side chain cleavages are highlighted in gray.

Results and Discussion

Figure 2 contains the (a) PSD at high MALDI laser power, (b) PSD at low MALDI laser power, and (c) 193-nm photofragment ion spectrum (at low MALDI laser power) for substance P $[M + H]^+$ ions. These data are summarized in Table 1 (the mass assignments in Table 1 are from a single measurement). At high MALDI laser power, a complete series of a-type, except a_9, fragment ions are observed. Note, however, that the

Table 1. Observed and calculated *m/z* for substance P fragment ions

Peptide fragment	Observed *m/z*	Calculated *m/z*	Error
P	70.07	70.07	0.00
a_1	129.12	129.11	0.01
a_2	226.19	226.17	0.02
a_3	354.31	354.26	0.05
a_4	451.46	451.31	0.15
a_5	579.49	579.37	0.12
a_6	707.39	707.43	0.04
a_7	854.51	854.50	0.01
a_8	1001.39	1001.57	0.18
a_{10}	1172.17	1171.67	0.50
b_{11}	1330.70	1330.71	0.01
$[M + H]^+$	1347.77	1347.74	0.03
		average	**0.09**

dominant a-type ions observed correspond to a_i-17 (probably NH_3). Ammonia loss reactions are especially abundant for ions that are thermally excited [9]; thus the presence of the a_i-17 at high laser powers possibly reflects a thermal component to the MALDI process. At low MALDI laser power, the abundance of PSD fragment ions is very low and very few sequence specific fragment ions are detected. The photofragment ion spectrum contains a complete series of sequence specific ions, and the most abundant ion series are the a_i ions. Note, however, that this spectrum also contains b_i- and y_i type as well as internal fragment ions. In addition, abundant side-chain cleavage ions are detected. The d_i ions are formed by loss of 42 mass units from leucine at position 10 and loss of 57 mass units from glutamine at positions 5 and 6 and loss of 57 mass units from lysine at position 3. The photofragment ion spectrum is very similar to that reported previously by Johnson et al. [10] obtained by CID using a tandem sector type mass analyzer.

Incorporation of photodissociation into MALDI-TOF MS improves the utility for peptide sequencing (manuscript in preparation) and also provides a solution for distinguishing isobaric residues, e.g., leucine and isoleucine. In addition, the increased abundances of sequence specific fragment ions by photodissociation may prove useful for sequencing of peptide mixtures where the abundance of PSD fragment ions is reduced [11], and for ions formed by electrospray ionization (ESI), where the abundance of fragment ions is reduced due to "cooling" of the ions by evaporative processes [12].

Another important aspect of photodissociation using MALDI-TOF is that the resolution for mass selection of the analyte ion is determined by the pulse characteristics of the laser. Although the mass resolution of the pulsed deflector lenses used to mass-select the analyte ions for PSD is relatively poor when compared to Fourier-transform ion cyclotron resonance (FTICR) MS and sector type tandem instruments, the narrow pulse widths for the photodissociation laser (3–6 ns) are sufficient for mass-selection of the ion that is photoexcited. Ions that are close in *m/z* ratio to the photoexcited ion do not absorb a photon and thus do not yield photofragment ions.

The overall mass measurement accuracy is limited by the intensity of the fragment ion profiles (see Table 1). Ion profiles with good signal-to-noise ratios are measured accurately ($<$0.1 u error) whereas low signal-to-noise profiles are not measured as accurately ($>$0.1 u error) [4]. Because of the relatively low intensity and low mass measurement accuracy for the a-type ions of lysine and glutamine, where both lose 57 u by side chain cleavage, it is not possible to distinguish between the side chains from the a- and/or d-type fragments. With increased signal-to-noise and multiple measurements, the difference of 0.04u could be elucidated using the a-type fragments and confirmed by the d-type fragments.

Data acquisition rates for excimer photodissociation can be quite high (200 Hz for the laser used in this study) and photofragment ion spectra can be acquired at high rates. The high data acquisition rate reduces the overall time required for data collection. In addition, the high data acquisition rates makes it possible to acquire the photofragment ion spectrum at a rate compatible with chromatographic sample introduction (peak widths of 100 ms to 1 s).

Conclusions

Photodissociation of substance P on a MALDI reflectron TOF results in a photofragment spectrum comparable to high energy CID on a tandem mass spectrometer. Differentiation of isobaric residues is possible due to the presence of d-type fragments appearing from side-chain losses.

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